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14. ABSTRACT Background: Due to the changing nature of war, there is a pressing need for new treatments for traumatic brain injury (TBI). The grantee previously found screened that the combination of minocycline (MINO) and N-acetyl cysteine (NAC) synergistically improved brain function when dosed one hour following closed cortical impact (CCI) in rats. The overall objective of this proposal is that MINO/NAC synergistically improves brain function following TBI. Four tasks will be done to achieve this objective: 1) Differing doses of MINO/NAC will be tested for the ability to improve behavior and histology following moderate CCI. 2) MINO/NAC was effective when dosed one hour after injury. Longer intervals between injury and drug dosing will be tested. 3) TBI is clinically heterogenous; MINO/NAC should restore brain function following an additional TBI model, lateral fluid percussion. 4) A restoration of cognitive function will be tested three months after CCI. The grantee has received both institutional and DoD approval for the animal experiments in this work and has trained a MD-Ph.D. candidate to perform these studies.						
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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	8
Reportable Outcomes.....	7
Conclusion.....	7
References.....	7
Appendices.....	8

Introduction

There is a pressing need for new treatments for TBI because of the increased frequency of head injuries during Operation Iraqi Freedom and Operation Enduring Freedom. Multidrug treatment is one of many therapeutic approaches that are being tested. The applicant previously performed a screen of drug pairs to treat TBI ("Multidrug treatment of traumatic brain injury", PT073028) from the Fiscal Year 2007 CDMRP program for Psychological Health/Traumatic Brain Injury. Drug pairs were screened that: (1) limited TBI as monotherapy in preclinical or early clinical trials and (2) have FDA approval for uses other than TBI. The drug screen used a novel behavioral assessment with high cognitive demand to find drug combinations that best restored brain function following moderate or mild closed cortical impact (CCI) (1). The applicant found that MINO and NAC improved both cognitive and histological outcomes following CCI (2). As monotherapy, MINO-treated rats learned, but had no 24-hour retention of an active avoidance task. NAC-treated rats were greatly impaired in both acquisition and retention. The combination of MINO plus NAC, given one hour after injury produced a large improvement in both acquisition and retention. NAC alone had no effect indicating a synergistic drug interaction with MINO in improving cognitive function. In addition, MINO plus NAC-treated rats had better sparing of both white and grey matter. These observations provide the justification for the central hypothesis that: MINO plus NAC synergistically improve brain function following TBI. MINO plus NAC has the potential to rapidly get a safe and effective combination therapy into clinical trials. The combination is likely to be safe. Both drugs have been used in the clinic for decades with well-known pharmacokinetics, pharmacodynamics and drug interactions. The current project contains key experiments needed to begin testing MINO plus NAC in the clinic.

Body

The statement of work for this project describes four tasks:

Task 1. Optimizing the dosing of MINO plus NAC in the CCI model of TBI.

Task 2. Evaluation of the Therapeutic Window of MINO plus NAC

Task 3. Determine the efficacy of MINO plus NAC in the lateral fluid percussion model of TBI

Task 4. Examination whether MINO plus NAC provide long-term behavior improvements.

Regulatory approval has been obtained for the animal work in this project by the SUNY-Downstate Institutional Animal Care and Use Committee as well as the USAMRMC Animal Care and Use Review Office. These approvals cover all four tasks in the statement of work.

In October, 2011 one key member of my research team, Samah Abdel-Baki, M.D. left the laboratory on short notice. Dr. Abdel-Baki was the first author on two previous studies of Dr. Bergold was recruited into a more lucrative position running a clinical research project (1, 2). In July 2011, Dr. Natalia Grin'kina, a post-doctoral fellow, and Ms. Margalit Haber, a Ph.D. graduate student with Dr. Bergold.

Dr. Grin'kina and Ms. Haber are in the process of becoming skilled in the the CCI model. To test their ability to perform the CCI surgery, rats were divided into two groups. In sham-CCI injury, a unilateral craniotomy (6.0 mm) was made in an anesthetized rat centered midway between lambda and bregma. The craniotomy exposed the dura without damaging the meninges or the cortex. An 5.0 mm diameter impactor tip of an electromagnetic contusion device (MyNeurolab, St. Louis, MO) was placed into the craniotomy. The impactor tip was then removed, a plastic plate covering the craniotomy was cemented (Grip Cement, Dentsply, York, PA) to the skull and the incision sutured closed.

Rats received the same treatment for moderate CCI injury as shams except after the impactor tip was placed into the craniotomy, it compressed the cortex to a depth of 2.5 mm at 4 m/s. Both

sham- and moderately-injured rats were returned to their home cages for one week followed by testing on a hierarchy of behavioral tasks developed by the awardee (1). Day 7 post-surgery consisted of open-field, and passive avoidance. Injured and sham-injured rats performed similarly on these tasks suggesting a lack of motor deficits, and similar ability to explore a novel environment and avoid shock. On the following day, rats had six ten-minute trials to learn an active avoidance task that required avoiding a stationary shock zone on a rotating arena. The number of entrances into the shock zone was the outcome that measured active place avoidance.

The moderately injured group did not decrease the number of entrances during training since there was no significant effect of trial (Figure 1, $F_{(5,35)}=0.87$, $p > 0.5$). This suggested that the injured rats did not learn the task. In the sham-injured group, four out of 10 of the sham-injured rats reduced the number of shock-zone entrances to fewer than 5 entrances on trial 6; the remaining 6 rats had more than 10 shock zone entrances on trial 6. Thus, this sham-injured group was further subdivided into two groups (sham, <10 ; sham, >5) based upon the number of trial 6 shock-zone entrances.

There were significant effects of trial in both groups (sham, <5 , ANOVA, $F_{(5,15)} = 5.0188$, $p < 0.01$; sham, >10 , $F_{(4,25)}=3.401$, $p < 0.02$) This suggests that, regardless of the number of entrances on the final trial both sham groups, acquired the task. Surprisingly, there was a significant effect of treatment among the three groups ($F_{(5,75)} = 2.848$, $p < 0.02$) suggesting that the two sham subgroups significantly differed in their ability to acquire the active avoidance task. These data further suggest that the active avoidance task has a sensitivity to detect injury that was not seen in previous studies. We are presently doing analyzing the histology of the three groups to further understand what occurs when the brain is damaged by craniotomy. The effect of craniotomy on the CCI has been recently discussed in a recent paper (3). Interestingly, the authors described histological changes occurring following craniotomy, but could not detect any behavioral deficits. In contrast behavioral deficits could be readily seen in sham-injured animals.

Task 1 Optimization of the dosing of MINO and NAC

Dosing of MINO (45mg/kg) plus NAC (150mg/kg) to rats one hour after moderate CCI has been previously shown to synergistically improved performance on the active place task (2). Task 1 will alter the amount of both MINO and NAC separately to obtain a more complete dose-response. Preliminary data from this task is shown in figure 2.

Rats received either sham- or moderate CCI. The moderate CCI group was divided into three group that were injected with saline or drugs one hour, one and two days after surgery. The three groups received saline, MINO 45 mg/kg plus NAC 150 mg/kg, or MINO 90 mg/kg plus NAC 150 mg/kg. The rats were to their home cages for 7 days and then were tested on the hierarchy of behavioral tests developed by the applicant (1). Total entrances into the shock zone were assessed in active place avoidance, the last test in the hierarchy. There was a strong group effect for treatment (ANOVA, $F_{(3, 21)} = 13.66$, $p < .0001$) with moderate CCI significantly increasing shock zone entrances ($p < 0.0001$, all pairwise comparisons analyzed using Bonefferoni post-hoc tes). MINO plus NAC at both doses significantly decreased shock zone entrances ($p < 0.005$), but there was no significant difference in the performance of animals receiving either dose ($p > 0.5$).

The histological analysis is ongoing, but representative images stained with Luxol fast blue from each group are provided. Luxol fast blue selectively stains myelin. The original protocol proposed analyzing myelin content in corpus callosum in coronal sections. More recent studies from my laboratory have shown that myelin loss is most close to the midline of the brain (Figure 3). With this better knowledge of the location of myelin loss induced by mild CCI, we are now analyzing myelin content in saggital sections. These studies are showing that, in addition to the corpus callosum,

mCCI selectively damages the dorsal and ventral hippocampal commissures as well as the anterior commissure.

The following conclusions can data repeat earlier finding that MINO plus NAC improves cognition after moderate CCI, but increasing the MINO concentration does provide a further increase in efficacy.

Development of a mouse model of closed cortical injury. There has been an ongoing problem in the CCI model because approximately 25% of the rats receiving sham injury showed varying amounts of impairment in the active place avoidance task. Many changes were made in the behavioral task were made to address this problem. These changes included:

- 1) Changing room and visual cues in the behavioral apparatus
- 2) Increasing the time for habituating rats to the laboratory, laboratory workers and behavioral apparatus
- 3) Testing sham-injured rats on other active avoidance set-ups at SUNY Downstate
- 4) Testing multiple drills for craniotomy
- 5) Purchasing Sprague-Dawley rats from alternative vendors
- 6) Placed rats on reversed day-night cycle to ensure that the rats were fully awake when tested during the day
- 7) Titrated amount of foot shock for each rat
- 8) Switched from the albino Sprague-Dawley rat strain to the non-albino Long-Evans strain.

None of the changes significantly decreased the number of rats that received sham-injury that showed impairments in the active place avoidance task. This suggested that the craniotomy used in the controlled cortical impact model produced sufficient brain injury to impair a subset of rats. As a result, Dr. Bergold decided to switch from the controlled cortical impact model to a closed head injury that does not require a craniotomy. Accompanying the change in the model was also changing the species used from rat to mouse. Mice were selected due to the thinner skull that increased the probability that a single blunt blow to the head could injure the brain.

Closed head injury was done on C57/BL6 mice (19-22g) by modifying the method of Mouzon, et al., (4). A baseline weight was obtained on all mice prior to sham-closed head injury (CHI) or sham-CHI. Deep anesthesia was induced with isoflurane (3% in oxygen (1.0 L/min)) and maintained via a nose cone and isoflurane (2% in oxygen (1.0 L/min)). The head of the mouse was fixed in a stereotaxic frame using soft foam pads (David Kopf, Tujunga, CA). Foam pads avoided the tendency of conventional ear bars to break the bones of the inner ear during the impact.

Rectal temperature was maintained at 36.5–37.5°C using a circulating warm water heating pad. Isoflurane anesthesia was maintained throughout the surgery. A cortical contusion was produced using an electromagnetic contusion device (Myneurolab, St. Louis, MO). A 5.0 mm diameter impactor tip was placed at a 10° angle 5mm off the midline and 2mm from the eyes of the mouse. Closed head injury was produced with by a single 6.3 m/s impact to the skull that compressed the skull either 1 or 3mm. After the impact, the mouse was removed from the device and allowed to recover in its home cage. A mouse was determined to have recovered from anesthesia if it has regaining its ability to right itself and ambulate. Sham-CHI mice underwent the same procedure without the impact. Mice were weighed every day after CHI or sham-CHI.

Behavioral assessments were done in a rectangular room (4m by 3m) in BSB 6-78 that contains a behavioral apparatus containing a 40-cm diameter metal disc with prominent visual landmarks on the walls. A computer tracked the position of the mouse using a computer controlled infrared Firewire camera mounted 1.2 m above the arena. The signal from the camera was analyzed by a spot-tracker

(BioSignal Group, Brooklyn, NY). The outer part of the arena was marked with a light emitting diode (LED). The positions of the mouse and the arena LED was determined every 33 ms and used to calculate the movement of the animal relative to the arena. In some determinations, the computer defined a 60° segment of the arena as a do-not-enter shock zone. A 500 ms shock was produced 500 ms after entry into the shock zone. Additional shocks were administered every 1.5 s until the mouse has vacated the shock zone. The shock consisted of a brief constant current (500 ms, 60 Hz, 0.2 mA) delivered through the grid floor. The number of times a mouse entered the shock zone was computed by Track Analysis software (Bio-Signal Group Corp., Brooklyn, NY).

Beginning 7 days after CHI or sham-CHI, mice was habituated to handling and the training environment. All training sessions were 10 minutes long with a 50 minute intertrial interval. Mice first had a pretraining session in consisting of a arena rotating 1rpm with the shock zone turned off. Total distance traveled was assessed. The mice then had 3 sessions with the shock zone turned on and number of shock zone entrances and the distance assessed.

Rats receiving sham-CHI had 8.5 ± 0.9 total entrances. This that was significantly fewer than the 23.6 ± 6 entrances observed in rats receiving CHI ($t_{15} p < 0.05$), Figure 3). These data indicate that CHI produces cognitive deficits in mice.

Mice were returned to their home cages after testing. Fourteen days after the injury or sham-injury mice were deeply anesthetized with 4% Isoflurane in 1.0L/min followed by transcardial infusion with paraformaldehyde (4% (w/v) in saline) for histological analysis

Key research accomplishments

Increasing the dose of MINO does not appear to change the efficacy of MINO plus NAC using number of entrance in the active avoidance task as an outcome measure.

A closed head injury model has been established in C57/Bl6 to be used for the remainder of this project.

Reportable outcomes

Dr. Bergold presented a portion of this work at the 2011 Experimental Biology Meeting at Washington DC 4/9/2011-4/13/2011 in the symposium: Traumatic Brain Injuries: Anatomical Challenges and Possibilities for Repair

Dr. Bergold presented a portion of this work at the 2011 Society for Neuroscience Meeting in Washington DC 11/12/2011-11/15/2011.

Dr. Bergold presented a portion of this work at the 2012 National Neurotrauma Society Meeting in Phoenix, AZ 7/22/2012-7/25/2012.

Conclusions

MINO (90mg/kg) plus NAC (150mg/kg) has improves cognition and memory no better than MINO (45 mg/kg) plus NAC (150mg/kg) .

After closed head injury, mice display behavioral deficits in the active place avoidance task similar to those seen after moderate CCI (Abdel-Baki, et al., 2010).

References

1. Abdel Baki, S.G., et al., Minocycline synergizes with N-acetylcysteine and improves cognition and memory following traumatic brain injury in rats. PLoS One, 2010. 5: e12490.
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Appendix

Figures

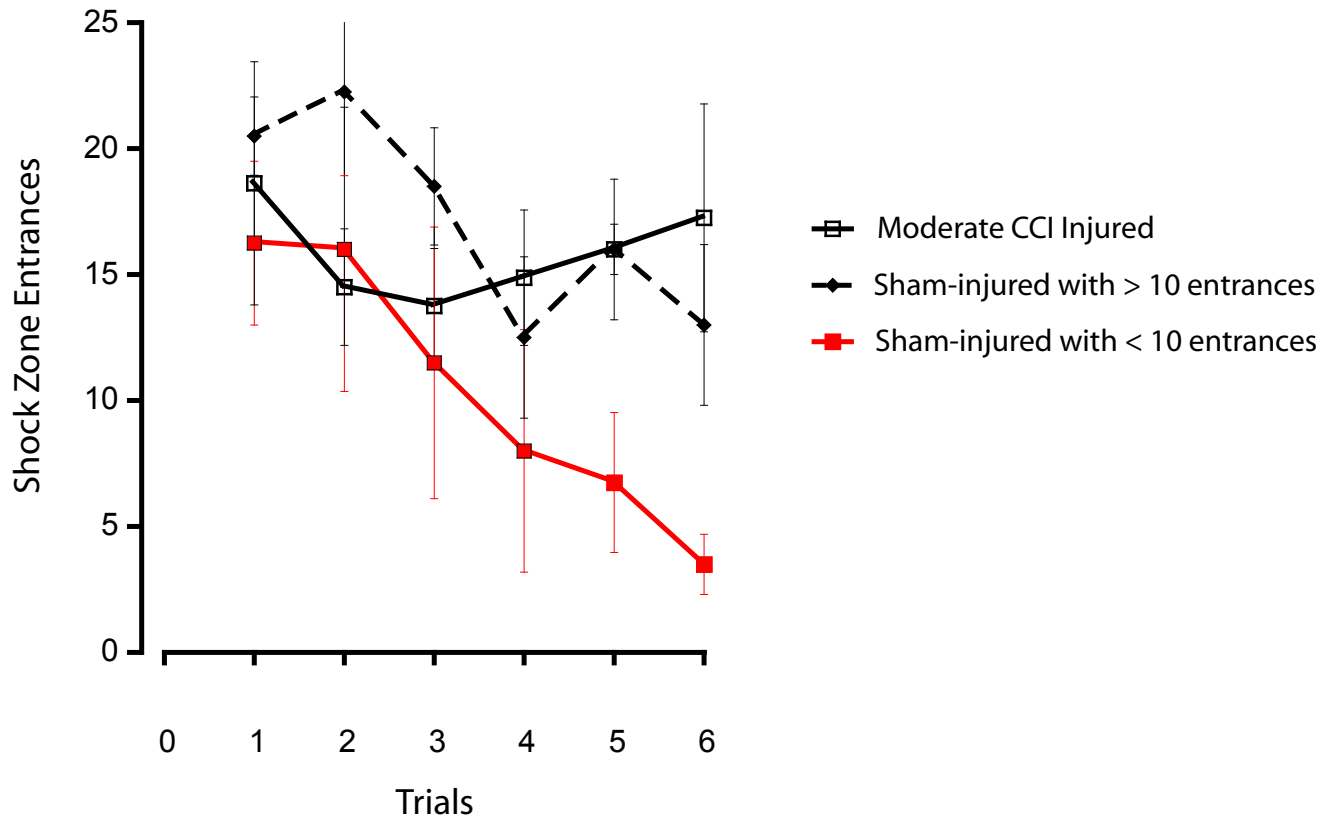
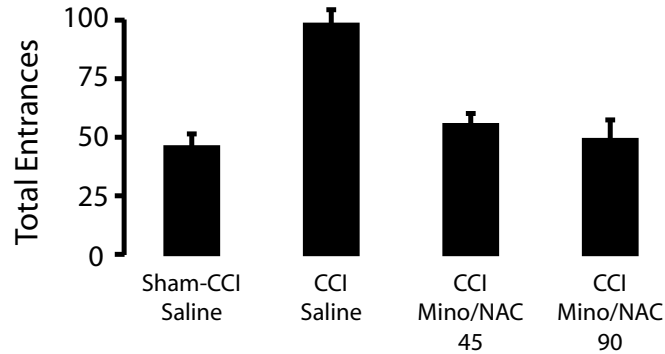
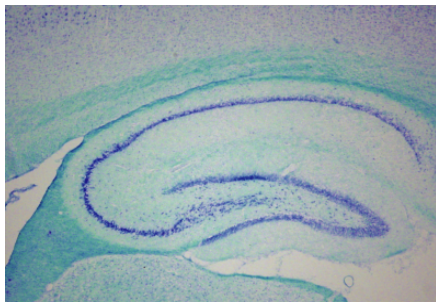


Figure 1 Two groups of sham-injured rats that have difference performance on the active avoidance task. Rats received either sham-CCI, consisting of a craniotomy; or moderate CCI. Rats were returned on their home cages for one week. They were then tested on open field and passive place avoidance. The following day the rats received six ten-minute trials of active place avoidance and the number of entrances in the shock zone assessed. The sham-injured group respond as a uniform group to the task was subdivided into two based on the number of entrances. The moderately injured group did not decrease the number of entrances during the six trials since there was no significant effect of trial ($F_{(5,35)}=0.87$, $p > 0.5$). This suggested that the injured rats could not acquire the task. In the sham-injured group, four out of 10 of the sham-injured rats reduced the number of shock-zone entrances to fewer than 5 entrances on trial 6; the remaining 6 rats had more than 10 shock zone entrances on trial 6. Thus, this sham-injured group was further subdivided into two groups (sham, >10; sham, >5) based upon the number of trial 6 shock-zone entrances. There were significant effects of trial in both groups (<10, ANOVA, $F_{(5,15)} = 5.0188$, $p < 0.01$; >10, $F_{(4,25)}=3.401$, $p < 0.02$) This suggests that both sham groups could acquire the task. Surprisingly, there was a significant effect of treatment among the three groups ($F_{(5,75)} = 2.848$, $p < 0.02$) suggesting that the two sham subgroups differed in their ability to acquire the active avoidance task.

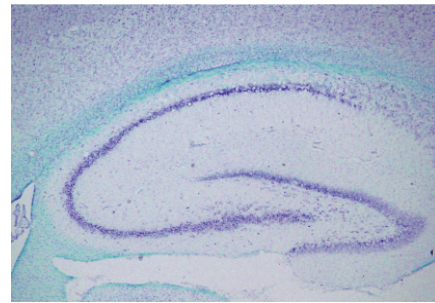
A) Behavioral Improvement produced by MINO/NAC



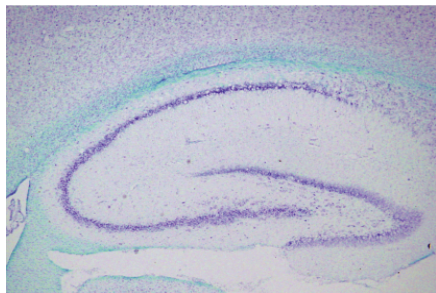
B) Myelin loss prevented by MINO/NAC



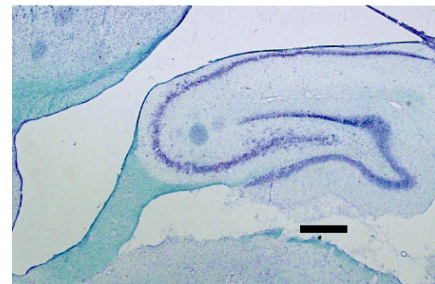
Sham-CCI Saline



Moderate CCI Mino 45 NAC 150



Moderate CCI Saline



Moderate CCI Mino 90 NAC 150

Figure 2 No change in behavioral improvement when the dosage of MINO is increased to 90 mg/kg from 45 mg/kg. Rats received either sham moderate CCI or moderate CCI. One hour later they began treatment with either saline MINO 45mg/kg or 90mg/kg plus NAC 150 mg/kg. Beginning seven days after surgery, rats received a battery of behavioral tasks that ended with massed active place avoidance. Panel A, The increase in shock zone entrances induced by CCI is prevented by MINO and NAC (ANOVA, $F_{3,21} = 13.66$, $p < .0001$, Significant difference from CCI-Saline, Sham-CCI saline, $p < 0.001$; CCI MINO 45/NAC, $p < 0.001$; CCI MINO 90/NAC, 0.001. There was no significant difference in the number of entrances between CCI MINO 45/NAC and CCI MINO 90/NAC ($p > 0.5$). These data suggest that there is no benefit taking a larger dose of MINO. Panel B, Myelin loss prevented by MINO/NAC. Fourteen days after the treatments described in panel A, rat brains were fixed, parasagittal sections were prepared and stained with cresyl violet and luxol fast blue. Scale bar 50 μ m.

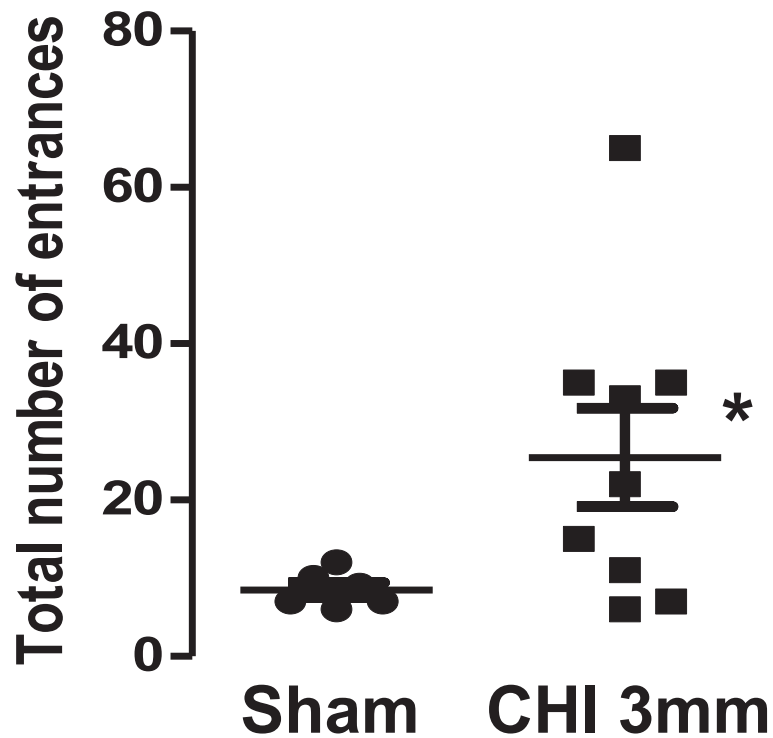


Figure 3 Increased shock zone entrances after closed head injury (CHI) in C57Bl6 mice. Mice received either sham-CHI (left) or CHI (right) The scatterplot shows the total number of shock zone entrances during three 10-minutes trials ability measured the ability to acquire the active place avoidance task. Mice receiving CHI had significantly more entrances than mice receiving sham-CCI ($*t_{15} < 0.05$). Also shown in the mean \pm SEM.

Abstracts of unpublished results

1) Abstract of a presentation by Dr. Bergold at the 2011 Experimental Biology Meeting in Washington DC 4/9/2011-4/13/2011 in the symposium: Traumatic Brain Injuries: Anatomical Challenges and Possibilities for Repair

Evaluation of clinically relevant models of traumatic brain injury

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Despite a large need, there are presently no treatments for traumatic brain injury (TBI). This study examined how the combination of minocycline and N-acetylcysteine (MINO/NAC) synergistically improved cognition and memory in a mild controlled cortical impact (mCCI) model of TBI. mCCI induced a long-lasting loss of axons and myelin. MINO/NAC promoted remyelination without affecting axonal loss. While MINO alone induced remyelination and provided some improvement in cognition, NAC was needed for further improvements in cognition and for restoration of long-term memory. Fingolimod, a recently approved drug to treat multiple sclerosis, appeared also to induce remyelination and partially improve cognition. The improvement of cognition by MINO or Fingolimod was less than with MINO/NAC. These studies suggest that remyelination is needed to improve cognition following mild TBI, but additional therapeutic targets are needed to restore long-term memory.

2) Abstract of a presentation by Dr. Bergold at the 2011 Society for Neuroscience Meeting in Washington DC

Minocycline and N-acetylcysteine promote remyelination after traumatic brain injury

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Abstract: Traumatic brain injury (TBI) may be better treated with drug combinations than single drugs. The combination of Minocycline (MINO, 45 mg/kg) and N-acetylcysteine (NAC, 150 mg/kg) were previously shown to improve cognition and memory in rats when dosed one hour after a controlled cortical impact (CCI) that models moderate TBI (Abdel Baki, et al., PLoS One, (2010)). We now report that MINO/NAC is also effective in limiting injury when dosed one hour after rats received a mild form of CCI (mCCI). Within 2 days after mCCI, activated astrocytes and microglia are seen in corpus callosum and other white matter tracts. Within 4 days, corpus callosum lost $28.5 \pm 7.9\%$ (n=4) of axons and $74.5 \pm 5.3\%$ (n=4) of myelin content as measured by Luxol fast blue dye binding. This myelin loss remains unchanged 10 days later. mCCI also produces behavioral deficits. Injured rats are no different than sham-injured rats in avoiding a shock zone in an active avoidance task, but are completely impaired the following day when the shock zone was shifted 180°. This impairment is still evident one month after mCCI. Treatment with the drug combination of MINO and NAC beginning one hour after mCCI significantly decreases astrocytic ($57.2 \pm 7.0\%$, ANOVA, $F(3,10) = 31.58$, $p < 0.0005$) and microglial activation ($88.9 \pm 8.0\%$, $F(3,10) = 11.9$, $p < 0.005$). The drugs have no effect on axon loss or myelin content or the loss of myelin basic protein. These data suggest that mCCI produces a rapid white matter injury that is not effected by the drugs. Fourteen days after injury, however, myelin content in corpus callosum was significantly increased $38.8 \pm 8.5\%$ ($F(3,11) = 16.35$,

p<0.005). The increase in myelin induced by MINO plus NAC after mCCI suggest that the drugs work by remyelination. Repair of white matter by MINO/NAC may underlie the ability of the drugs to improve cognition and memory after mCCI.

3) Abstract of a presentation at the 2012 National Neurotrauma Society Meeting in Phoenix, AZ 7/22/2012-7/25/2012.

Minocycline and N-acetylcysteine modulates neuroinflammation and produces remyelination following controlled cortical impact

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Keywords therapeutics, white matter, repair, microglial activation, behavior

Following controlled cortical impact (CCI), MINO plus NAC (MINO/NAC) remyelinates white matter and improves both cognition and memory. We are testing whether changing the pattern of microglial activation by MINO/NAC induces remyelination. Improvement in cognition and memory may result from white matter repair.

Sprague-Dawley rats received either sham- or mild CCI (Abdel-Baki, et al., 2009) and treated at one hour and one day with MINO/NAC. Parasagittal sections were prepared 2 days after surgery that were stained with MHCII or CD68 that stains proinflammatory M1 microglia, arginase-1 that stains anti-inflammatory M2 microglia, or Iba-1 that stains both M1 and M2. M1 and M2 microglia were assessed in corpus callosum, dorsal or ventral hippocampal commissure (VHC) and anterior commissure. Cerebellar white matter acted as a control.

MINO/NAC also improved performance on an active place avoidance task. The hippocampal commissures are likely needed since task acquisition requires both hippocampi. Demyelination following CCI may impair or block commissural neural transmission. Stereotaxic lysolecithin injection produces a 2-week cycle of localized demyelination-remyelination. The VHC was injected with lysolecithin or saline (0.3ul, 1% w/v) followed by behavioral assessment.

Iba-1+ cells increased in corpus callosum in saline-treated rats two days after CCI. MINO/NAC treatment further increased the number of Iba-1+ cells. An increase in MHCII or CD68 expressing cells in injured saline-treated rats was not seen in rats treated with MINO/NAC. Examination of M2 markers is ongoing.

Saline-treated rats acquired the active place avoidance task four days after VHC injection. In contrast, rats injected with lysolecithin were completely impaired. After training, rats were sacrificed and myelin content assessed in the VHC. Saline-injected rats showed no change in VHC myelin content as seen by luxol fast blue staining. Lysolecithin-injected rats showed less myelin suggesting a localized demyelination. Examination of the VHC 2 weeks after injection showed no change in myelin content in saline-injected rats. In contrast, the reduced myelin content seen at 4 days after lysolecithin injection rats was significantly increased 2 weeks after injection. These data suggest a cycle of demyelination-remyelination induced by lysolecithin injection. Behavioral assessment is ongoing of rats 2 weeks after saline or lysolecithin injection.

These data suggest that MINO/NAC increases the number of activated microglia in damaged white matter regions after CCI. These microglia do not express M1 markers suggesting that they may have a M2 phenotype. Increased M2 microglia have been implicated in remyelination suggesting that MINO/NAC may repair white matter, in part, by altering the pattern of microglial activation following CCI.

Stereotaxic injections of saline and lysolecithin have suggested a key role of the VHC in the acquisition of the active place avoidance task. This is consistent with previous observations showing a need for both hippocampi. Commissural white matter is particularly vulnerable to TBI and in animal models of TBI. Taken together, these studies show the importance of white matter damage and repair. Drugs that target white matter such as MINO/NAC in rodents may also show efficacy in clinical TBI that also display white matter damage.

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